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ORIGINAL ARTICLE

Contribution of JAK2 mutations to T-cell lymphoblastic lymphoma development

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The JAK-STAT pathway has a substantial role in lymphoid precursor cell proliferation, survival and differentiation. Nonetheless, the contribution of JAK2 to T-cell lymphoblastic lymphoma (T-LBL) development remains poorly understood. We have identified one activating TEL-JAK2 translocation and four missense mutations accumulated in 2 out of 16 T-LBL samples. Two of them are novel *JAK2* mutations and the other two are reported for the first time in T-LBL. Notably, R683G and I682T might have arisen owing to RNA editing. Mutated samples showed different mutated transcripts suggesting sub-clonal heterogeneity. Functional approaches revealed that two *JAK2* mutations (H574R and R683G) constitutively activate JAK-STAT signaling in γ2A cells and can drive the proliferation of BaF3-EpoR cytokine-dependent cell line. In addition, aberrant hypermethylation of *SOCS3* might contribute to enhance the activation of JAK-STAT signaling. Of utmost interest is that primary T-LBL samples harboring *JAK2* mutations exhibited increased expression of *LMO2*, suggesting a mechanistic link between *JAK2* mutations and the expression of *LMO2*, which was confirmed for the four missense mutations in transfected γ2A cells. We therefore propose that active *JAK2* contribute to T-LBL development by two different mechanisms, and that the use of pan-JAK inhibitors in combination with epigenetic drugs should be considered in future treatments.

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INTRODUCTION

Precursor T-cell lymphoblastic neoplasms are aggressive hematological malignancies consisting of small- to medium-sized blast cells, which mainly develop in children (in particular adolescent males) but can also affect adults. Most often, these malignancies manifest with extensive marrow and blood affectation (called acute T-cell lymphoblastic leukemia, T-ALL), and less commonly as a mass lesion in the thymus/anterior mediastinum or in lymph nodes, with less than 25% marrow blasts (then called T-cell lymphoblastic lymphoma, T-LBL).[1] The molecular basis of these neoplasms has been well established in T-ALL,[2,3] but to a lesser extent in T-LBL.[4,5]

It is well known that the JAK-STAT pathway has a substantial role in lymphoid precursor cell proliferation, survival and differentiation.[6] Thus, it was not surprising that hematological malignancies had been associated with aberrant JAK-STAT signaling transduction.[7] In fact, several authors reported activating mutations of two members of the Janus family of tyrosine kinases, *JAK1* (4–18%) and *JAK3* (5%), in a fraction of T-ALLs.[8–11] Notwithstanding, aberrant JAK-STAT signaling transduction was first reported in T-ALL exhibiting the *t*(9;12) (p24;p13) translocation, a rare rearrangement resulting in the constitutively active TEL-JAK2 kinase fusion oncoprotein.[12] This fact, together with the discovery of activating mutations of *JAK2* in other hematological malignancies, such as myeloproliferative neoplasms,[13,14] in particular the gain-of-function substitution of valine to phenylalanine at codon 617 at the pseudokinase domain of JAK2 found in patients with polycythemia vera,[15,16] showed the clinical relevance of this gene given more attention on the grounds of potential involvement in T-cell lymphoblastic leukemia/lymphoma. Whether acquisition of *JAK2* mutations is an important event in the pathogenesis of these neoplasms is still unclear. To explore this possibility, we performed a mutational screening of this gene in a new sample series of T-LBLs.

As JAK2 mediates crucial physiological events, its activity is tightly regulated through additional mechanisms. It is well known that dephosphorylation of JAK2 by protein-tyrosine-phosphatases, such as SHP1,[17] and recruitment of negative regulators, such as suppressors of cytokine signaling (SOCS) proteins, are involved in the downregulation of JAK-STAT signaling by different mechanisms.[7,18] SOCS proteins are silenced in many tumors by hypermethylation of CpG islands at their promoters. Reduced *SOCS1* expression is a frequent event in certain types of lymphoma and myelodysplastic syndrome,[19,20] including patients who harbor an activating *JAK2* mutation.[21] Another member of

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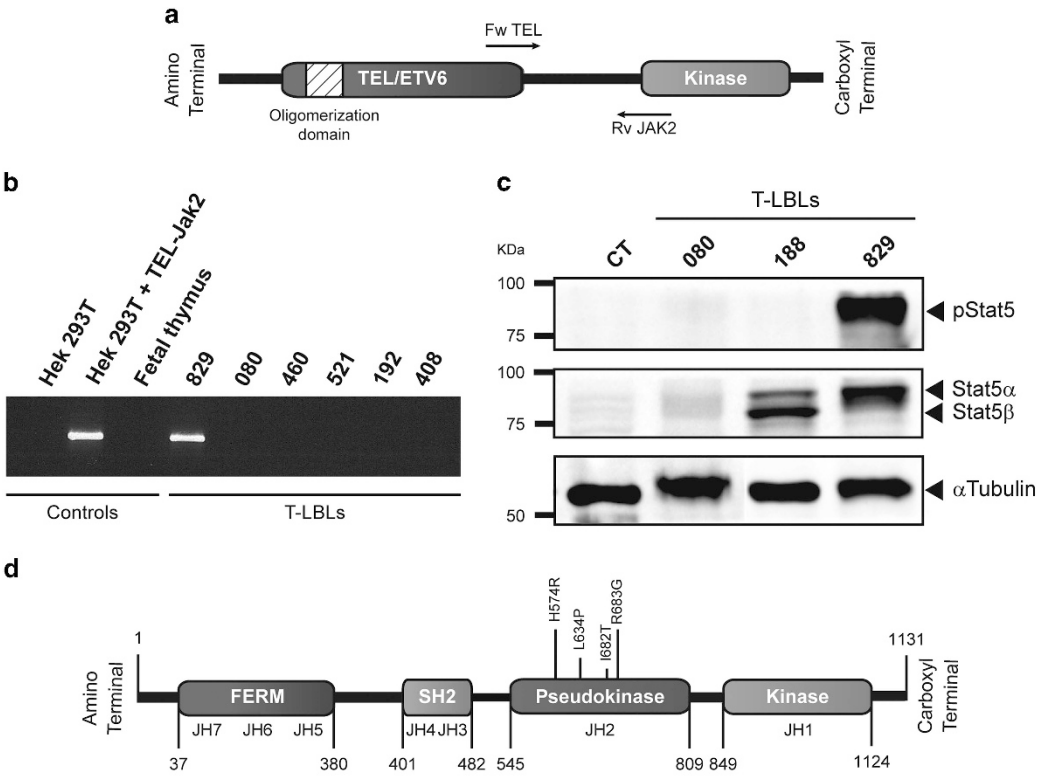
the same protein family, SOCS3, binds and directly inhibits the catalytic domains of JAK1 and JAK2 proteins,[22] and reduced expression of SOCS3 has been reported in various human cancers associated with constitutive STAT3 activation.[23] To assess for the possible contribution of these negative regulators in activating the JAK-STAT pathway, we studied the methylation density and the distribution of methylated CpG sites at the promoters of *SHP1*, *SOCS1* and *SOCS3*.

On the other hand, recent studies have demonstrated a nuclear role for JAK2 activation in regulation of LMO2.[24] LMO2 is essential for normal hematopoiesis development and has been implicated as a potent oncogene in a subset of T-cell lymphoblastic leukemia.[25–28] Transfection of *JAK2* into JAK2-null γ2A cells demonstrated the nuclear localization of JAK2 to induce an epigenetic control of *LMO2* expression. In line with this observation, *LMO2* has been reported as one of the genes whose messenger RNA levels were most decreased by inhibition of JAK2.[24] Therefore, we also explored whether *JAK2* activating mutations identified in our sample series of T-LBLs may be related to a *LMO2* increase in this type of lymphoma.

MATERIALS AND METHODS

Human sample collection

Sixteen human T-LBL samples (eight pediatric and eight from adult patients), and four thymuses of human fetus without pathology, were provided by the following Biobanks integrated in the Spanish Hospital Biobanks Network (RetBioH; www.redbiobancos.es): i+12 Biobank in the *Hospital 12 de Octubre* (samples 226 and 829), *Hospital Universitario Ramón y Cajal-IRYCIS* Biobank (sample 346), *Hospital Virgen de la Salud* Biobank in Toledo Medical Center (samples 188 and 192), *Complejo Hospitalario de Vigo* Biobank (sample 521), *Hospital General Universitario Gregorio Marañón* Biobank (samples 038, 080, 135, 154, 460 and 685), Hospital de La Paz Biobank (samples 238 and 408) and *Complejo Hospitalario Universitario de Santiago* Biobank (samples 516 and 731-4). The samples and associated data were processed and released following standard operation procedures with appropriate approval by Ethical and Scientific Committees. Lymphomas were diagnosed according to World Health Organization Classification of Hematological Malignancies and recommendations from the European childhood lymphoma pathology panel.[29,30] When adequate material was available, additional markers were added to improve the subclassification of T-LBLs (Supplementary Table S1). Institutional review board approval was obtained for these studies (reference CEI 31-773). The participants provided written informed consent in accordance with the Declaration of Helsinki.



JAK2 MUTATIONS					
Sample	Transcript 1	Transcript 2	Transcript 3	Transcript 4	Transcript 5
460	Wild type	c.1755A>G p.K585K	c.1901T>C p.L634P	c.2045T>C p.I682T	c.2047A>G p.R683G
734	Wild type	c.1721A>G p.H574R	c.1758A>G p.A586A		

Figure 1. TEL-JAK2 translocation and mutational analysis of JAK2. (**a**) Schematic representation of the fusion protein resulting from the t(9;12)(p24;p13) translocation. (**b**) PCR amplification of cDNA from a representation of the samples and HEK293T transfected with TEL-JAK2 positive control, using primers covering the breakpoint of this translocation. (**c**) Western blot analysis of STAT5 and phospho-Y694-STAT5 in T-LBLs and fetal thymus as control (CT). (**d**) Schematic representation of JAK2 protein showing all validated mutations at pseudokinase domain. All mutations were found in two T-LBL samples, both of them exhibiting several transcript variants.

Screening for TEL-JAK2 translocation

Identification of TEL-JAK2 translocations was performed by PCR amplification of cDNA using specific primers[12,31] (Figure 1; Supplementary Table S2). To obtain a positive control, we transfected HEK 293T cells (American Type Culture Collection, ATCC; Manassas, VA, USA) with *TEL-JAK2* cDNA cloned into pcDNA3 expression vector (a gift from Dr. Olivier Bernard at the Institute National de la Santé et de la Recherche Médicale, France) using Lipofectamine Reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA).

Mutational analysis of JAK2

Reverse transcription was carried out using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) followed by PCR with the Fast Start High Fidelity PCR System (Roche Applied Science, Basel, Switzerland). PCR fragments expanding mutational hot spots of this gene from exons 12 to 16 were cloned into pGEM-T Easy Vector (Promega, Life Technologies, Carlsbad, CA, USA). Mutation analysis of *JAK2* was performed in 20 cDNA clones of each tumor sample by direct sequencing. The presence of specific mutations at either genomic or transcriptomic level was validated by pyrosequencing analysis. The set of primers for PCR amplification were designed with Vector NTI software (Life Technologies Corporation), and primers for pyrosequencing were designed using the specific software PyroMark assay design (version 2.0.01.15). Pyrosequencing was performed using PyroMark Q24 reagents, and vacuum prep workstation, equipment and software (Qiagen, Valencia, CA, USA). Primer sequences are indicated in Supplementary Table S2.

Gene expression analysis

The expression of *JAK2* and *LMO2* genes was quantified at the transcript level by real-time quantitative RT-PCR from total RNA using a two-step procedure: first the Transcriptor First Strand cDNA Synthesis Kit, then the LightCycler FastStart DNA Master SYBR Green I (Roche). Expression values of *β2μG* (beta-2-microglobulin) were used as reference. Primers are described in Supplementary Table S2.

Protein expression was determined by western blot using the following primary antibodies: Jak2 (D2E12) Rabbit mAb (Cell Signaling Technology, Inc., Danvers, MA, USA), Phospho-Jak2 Tyr1007/1008 (C80C3) Rabbit mAb (Cell Signaling), Stat5 Rabbit (Cell Signaling), Stat5 (pY694) (BD Biosciences, San Jose, CA, USA), SOCS3 (ab16030) (Abcam, Cambridge, UK), β-Actin mouse (AC-15) (Sigma, Saint Louis, MO, USA) and α-Tubulin mouse (DM1A) (Sigma). Two secondary antibodies were used: anti-mouse IgG HRP-linked antibody (Cell Signaling), and anti-rabbit IgG HRP-linked antibody (Cell Signaling).

To investigate SOCS3 phosphorylation state (pSOCS3), first, we immunoprecipated SOCS3 using anti-SOCS3 antibody (ab3693) (Abcam), and second, we analyzed pSOCS3 expression using anti-phosphotyrosine (clone 4G10) (Upstate Biotecnology, Lake Placid, NY, USA), following the protocol described by Elliot *et al.*[32] Mouse TrueBlot ULTRA (Anti-Mouse Ig HRP) (Rockland, Limerick, PA, USA) was as used as the secondary antibody.

Immunohistochemistry

JAK2 expression was detected by immunohistochemistry in fresh-frozen tissue sections of two samples (238 and 460) using rabbit anti-JAK2, (D2E12#3230) (Cell signalling Technology) as the primary antibody. Polyclonal Goat Anti-Rabbit Immunoglobulin/HRP P0448 (Dako, Hamburg, Germany) and Liquid DAB+Substrate Chromogen System K3467 (Dako) were applied for visualization. Sections were counterstained with hematoxylin (Sigma) and observed on an Axiovert 200 inverted microscope (Zeiss, Göttingen, Germany). Images were acquired with a color ccd monochrome camera (SPOT RT Slid) by Devices Zeiss MTB Microscope software and processed with *Fiji-Image J* free software (http://fiji.sc; National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence

Subcellular distribution of JAK2 protein was studied by immunofluorescence in transfected γ2A cells (JAK2-deficient human fibrosarcoma cells provided by Dr. Ana Costa Pereira, Imperial College, London) using rabbit anti-JAK2, (D2E12) Rabbit mAb (Cell Signaling) as the primary antibody and anti-rabbit Alex Fluor-488 (Invitrogen) as the secondary antibody. Images were acquired with a Confocal LSM510 Zeiss microscope and processed with *Fiji-Imaje J* free software (http://fiji.sc; National Institutes of Health).

Site-directed mutagenesis

Human PCR fragment from *JAK2*-cDNA was amplified using the primers indicated in Supplementary Table S2 and cloned into the pcDNA3 vector (Invitrogen). All JAK2 mutants were generated by PCR reactions with QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA).

In addition, JAK2-H574R and JAK2-R683G mutants were generated in pEZ-Lv201 vector expressing human JAK2 (Genecopoeia, Rockville, MD, USA) by PCR reactions with QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies).

Dual luciferase assays

The functional activity of *JAK2* mutants was measured in γ2A cells by dual luciferase assays (Promega) with the STAT5 luciferase reporter pGL4.52 [*luc2P*/STAT5 RE/Hygro] vector (Promega).[33] Cells were seeded in 12-well plates overnight and transiently co-transfected using siLentFect (Bio-Rad, Hercules, CA, USA) with pGL4.52[*luc2P*/STAT5 RE/Hygro] vector, together with *STAT5A-pCMV6-XL4* and wild-type or mutant JAK2-pcDNA3 constructs. pRLTK was always co-transfected as an internal control. The cells were lysed in passive lysis buffer 24 h after transfection and luminescence was recorded on a single tube luminometer (Sirius luminometer; Berthold, Munich, Germany) following the manufacturer's instructions.

Proliferation assays

Murine cytokine-dependent cells expressing erythropoietin (Epo) receptor (BaF3-EpoR cells) were kindly gifted by Dr David M. Weinstock (Dana-Farber Cancer Institute, Boston, MA, USA) and Dr Charles Mullighan (St. Jude Children´s Research Hospital, Memphis, TN, USA). BaF3-EpoR cells were transduced with lentiviral pEZ-Lv201 empty vector, pEZ-Lv201 JAK2 WT, pEZ-Lv201 JAK2 H574R and pEZ-Lv201 JAK2 R683G and selected for stable expression of the constructs. Cells were sorted by FACS (FACSCVantage SE; BD Biosciences) according to comparable levels of GFP expression. Sorted cells were washed and seeded at 1×10^5 cells/ml in medium deprived of Epo. Viable cells were counted every 24 h for four consecutive days by trypan blue exclusion and the TC10 Automated Cell Counter (Bio-Rad).

Methylation analysis of SHP1, SOCS1 and SOCS3 genes

Methylation-specific PCR was performed according to conventional procedures based on bisulfite-mediated conversion of unmethylated cytosines to uracil; genomic DNA (500 ng) was modified with sodium bisulfite using kit Zymo EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) in agreement with the instructions of the manufacturer. The set of primers for PCR amplification and sequencing were designed using the Methyl primer Express software (Applied Biosystems, Foster City, CA, USA). PCR fragments were cloned into pGEM-TEasy Vector (Promega). A minimum of 8–10 colonies per sample was sequenced. The methylation status of every CpG was determined using the Sequencing Analysis 5.2 Software from Applied Biosystems. Primers and conditions are indicated in Supplementary Table S2. Some specific primers were as previously described.[34–36]

Epigenetic studies of SOCS3 in cell lines

To induce DNA demethylation, Jurkat and Molt-4 cells (German Collection of Microorganisms and Cell Cutures; DSMZ, Braunschweig, Germany) were grown in the presence of 2.5 μM 5'-aza-cytidine (Sigma). The histone deacetylase inhibitor trichostatin A (Sigma) was used at 1.0 μM and added to the cultured cells for 24 h. Combined treatment with 5'-aza-cytidine and trichostatin A was performed by incubating the cells with 5'-aza-cytidine for 72 h, followed by a 24-h exposure to trichostatin A. Expression levels of *SOCS3* were quantified by real-time quantitative RT-PCR. Specific primers are indicated in Supplementary Table S2.

Statistical analyses

The Shapiro-Wilk test was used to check expression data sets for normality, and the Levene test was used for homogeneity of variances. Student's *t*-test was used to compare gene expression mean values in controls and experimental groups with Bonferroni correction for multiple comparisons. These statistical analyses were performed using R software. All data reported represent at least three independent replicates.

RESULTS

One adult Pro-T-LBL sample exhibited activating TEL-JAK2 *t*(9;12)(p24;p13) translocation

TEL-JAK2 fusion events have been associated with T-ALL, as well as with atypical chronic myeloid leukemia.[12] With the aim of looking for TEL-JAK2 *t*(9;12)(p24;p13) translocation, we amplified cDNA from all 16 lymphomas (Supplementary Table S1) using appropriate primers (Supplementary Table S2). Amplification from HEK 293T cells transfected with a TEL-JAK2-cDNA pcDNA3 construct (resulting from a fusion between the 3´part of the kinase (JH1) domain of JAK2 and the 5´region of Translocation Ets Leukemia (*TEL)* gene,[12] provided us with a positive control. Only one TEL-JAK2 translocation was detected in only one patient with adult Pro-T lymphoblastic lymphoma (829) (Figures 1a and b).

To determine the functional consequences of this translocation, the status of STAT5 tyrosine phosphorylation was tested in selected samples using an activation-specific STAT5 antibody that selectively recognizes STAT5 phosphorylated at tyrosine 694 (pSTAT5). The sample harboring the TEL-JAK2 fusion protein exhibits the highest levels of pSTAT5, suggesting constitutive activation of the JAK-STAT signaling pathway. Stripping and reprobing the membrane with an antibody that recognized STAT5A and B showed differential STAT5 protein expression for each tumor (Figure 1c).

Four missense substitutions in JAK2 were validated in two pediatric T-LBL samples that exhibit sub-clonal heterogeneity

As activating mutations of *JAK2* have been reported in other hematological malignancies,[13–16] we performed a mutational screening of this gene in our sample series of T-LBLs. A comparative study of the *JAK2*-cDNA sequence encoding exons 12 to 16, which cover the pseudokinase domain of *JAK2* gene, revealed eight sequence variants (single nucleotide variants) that accumulate in two out of eight pediatric patients (460 and 734; 25%). Six of these variants were missense mutations and, to our knowledge, four of them have not been previously reported for *JAK2* (E549K, I682T, H574R and K558R). Mutation L634P has been reported in large intestine carcinoma (Catalogue of Somatic Mutations in Cancer, COSMIC database; Id. COSM1462578); mutation R683G has been reported in childhood B-precursor acute lymphoblastic leukemia associated with Down's syndrome[37–41] (Figure 1d; Supplementary Figure S1).

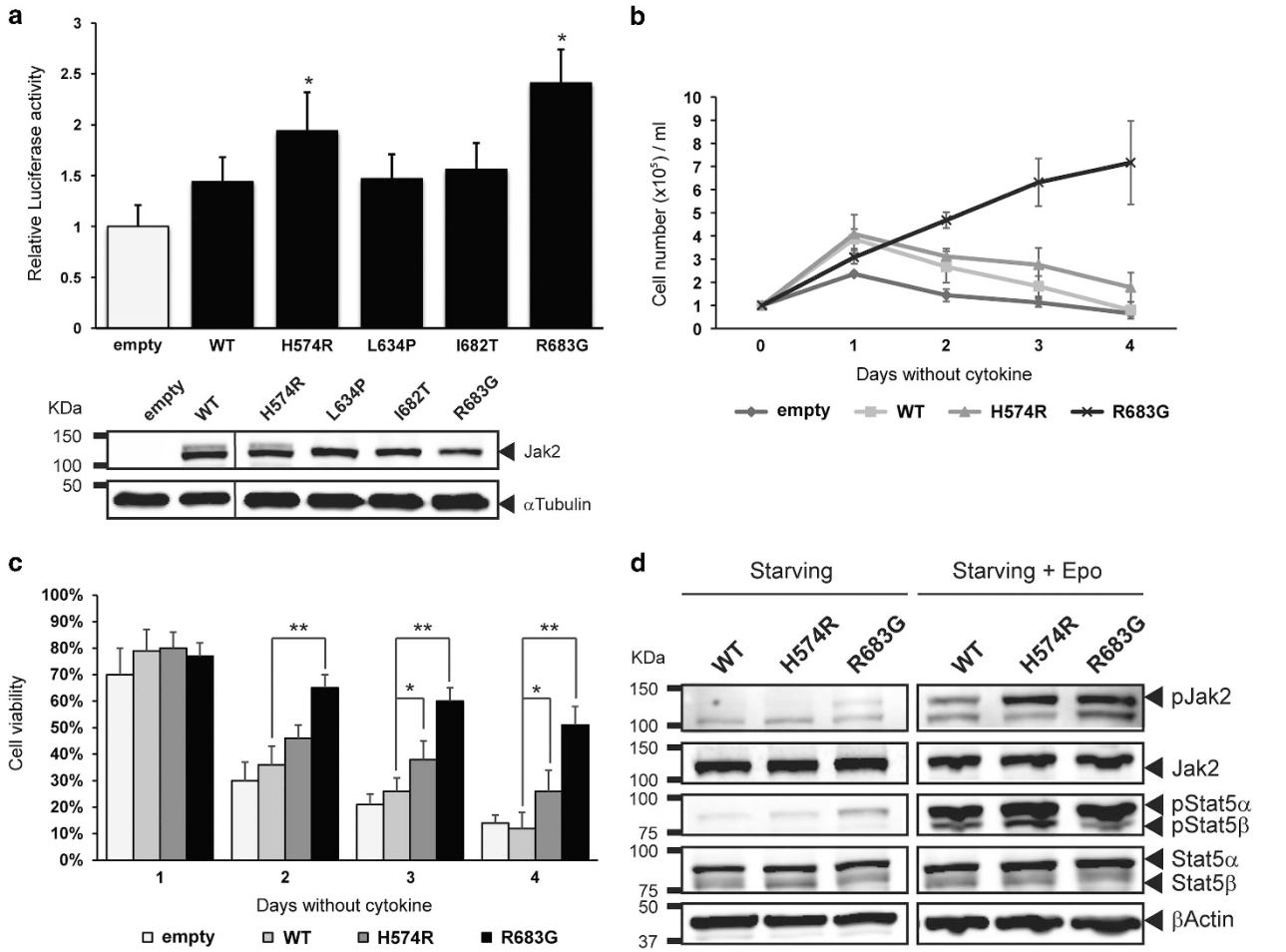


Figure 2. Effects of the JAK2 mutations in inducing JAK/STAT pathway activation and in driving cytokine independence cell proliferation. (**a**) JAK2-driven STAT5 transcriptional activity, measured by luciferase assay in γ2A cells transiently transfected with wild-type or four different mutant *JAK2*, or empty vector as a control. A representative western blot of JAK2 can be seen under the bar chart. Error bars represent the standard deviation (s.d.); $*P < 0.01$. (**b** and **c**) BaF3-EpoR cells stably expressing WT-JAK2, H574R-JAK2 or R683G-JAK2 were washed and seeded at 1×10^5 cells/ml in medium deprived of Epo. Viable and total cells were counted every 24 h for four consecutive days. (**b**) A graphical view of cell proliferation for each cell line. Error bars represent the s.d.; $*P < 0.05$ and $** P < 0.01$. (**c**) Bar representation of cell viability in percentage, indicating the significance between JAK2 variants within time points. (**d**) Representative western blot analysis of sorted BaF3-EpoR cells stably expressing WT-JAK2, H574R-JAK2 or R683G-JAK2. Cells were maintained for 24 h in medium without Epo (*Starving*) and then stimulated or not for 20 min with 1 U/ml of Epo (*Starving+Epo*). Total levels or phosphorylation of JAK2 and STAT5 were analyzed by incubation with their specific antibodies. Error bars represent the s.d.; $*P < 0.05$ and $**P < 0.01$.

To further demonstrate the presence and frequency of these mutations at the transcriptomic and genomic levels, we performed pyrosequencing as a more sensitive targeted-deep-sequencing approach to analyze PCR-amplified fragments covering mutation sites. Remarkably, we could validate the presence of four mutants (H574R, L634P, I682T and R683G). In case of L634P, cDNA analysis could not be possible because of the poor state of cDNA material. Notably, two of the confirmed mutations (R683G and I682T) were not found at the genomic level, suggesting that they have arisen by means of RNA-editing processes. It should be stressed that pyrosequencing from cDNA was performed on independent cDNA syntheses of samples 460 and 734.

Among the four confirmed mutations, the most frequent was R683G (at tumor 460), which appeared in 14.28% of cDNA clones. However, pyrosequencing data revealed that the most common mutation was H574R (at tumor 734), which appeared in 20.51% of the genomes and in 21.6% of the JAK2 transcripts (Supplementary Figure S1).

It should be noted that each mutation appears as unique to a specific cDNA clone ('private' mutations). Furthermore, because

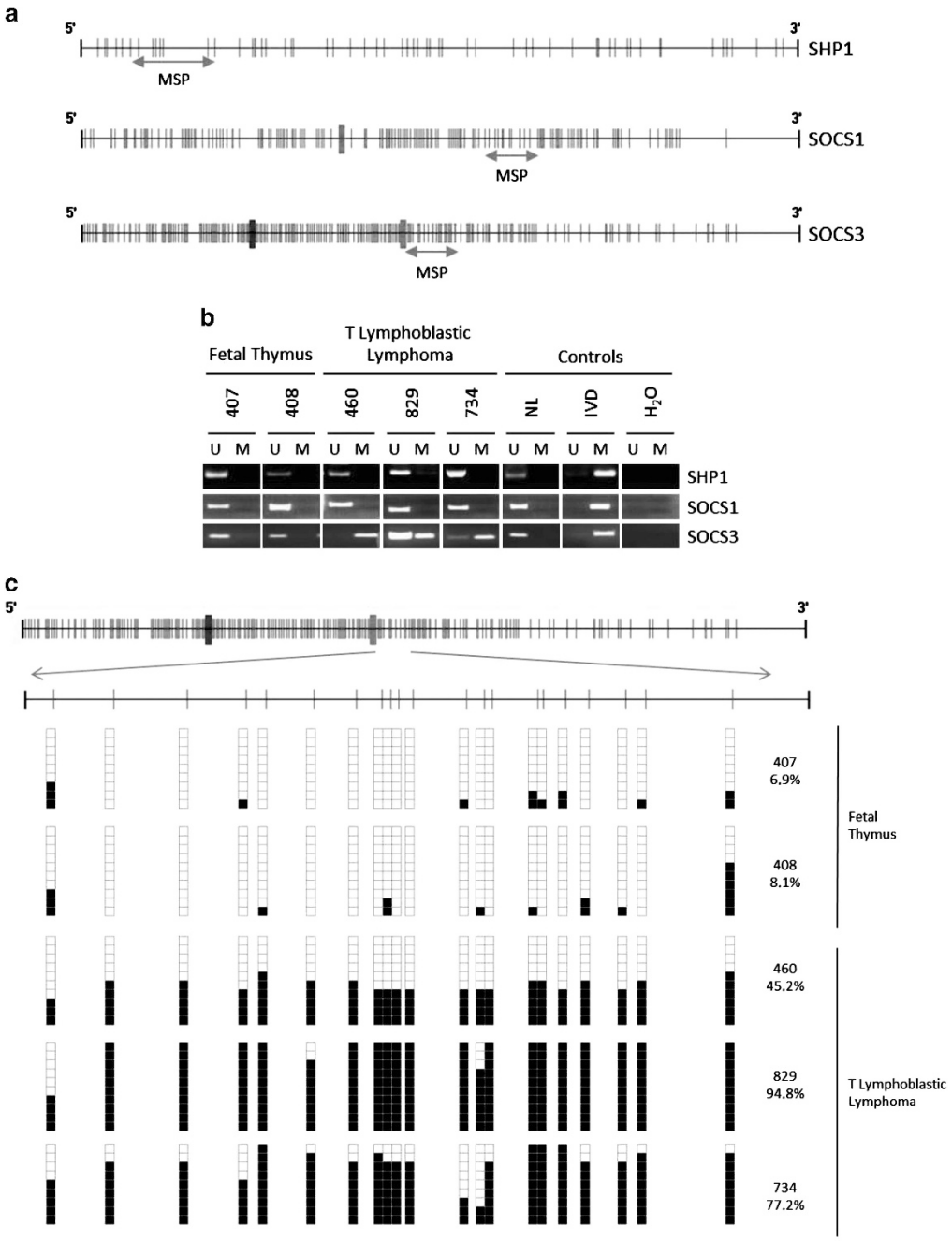


Figure 3. Methylation-specific PCR (MSP) for *SHP1*, *SOCS1* and *SOCS3* genes and methylation density at the *SOCS3* promoter, in T-LBLs with mutant JAK2. (**a**) Schematic depiction of the CpG-island around the transcription start site of the genes (long black arrow). Short vertical lines represent CpG dinucleotides. (**b**) MSP analysis. The presence of a PCR band under lanes M or U indicated methylated or unmethylated CpG islands. Normal lymphocytes (NL) and *in vitro* methylated DNA (IVD) were used as a positive control. (**c**) Methylation profile of SOCS3 in two fetal thymuses and T-LBLs harboring JAK2 genetic alterations. The presence of methylated (black squares) or unmethylated (white squares) CpG sites is indicated in 10 sequenced clones for every tumor. Methylation density is indicated as the percentage of methylated sites.

the two mutated T-LBL samples exhibited three or more sequence variants, it would be reasonable to think of sub-clonal heterogeneity within these tumors.

Functional significance of JAK2 mutants

To assist in the selection of confirmed mutations, we used two mutation effect prediction algorithms: PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.bii.a-star.edu.sg/). Because both algorithms returned predictions that showed some discrepancies, we selected all four missense mutations for functional analyses (Supplementary Table S3). To assess whether JAK2 mutants are able to constitutively activate the JAK-STAT signaling pathway, we performed dual luciferase assays in γ2A JAK2-deficient cells. Functional assays measuring luminescence in γ2A cells co-transfected with *JAK2*-mutant constructs and STAT5-luciferase reporter, revealed a tendency for JAK2 mutant variants to drive STAT5-mediated luciferase to a higher expression levels than the wild-type construct, this increase being statistically significant for two *JAK2* mutants: R683G from lymphoma 460 and H574R from lymphoma 734. As there are no apparent differences in JAK2-protein levels between wild-type and JAK2 variants, this factor should not disturb the interpretation of the data and their statistical significance (Figure 2a). It would therefore be reasonable to think that at least these two mutations are able to constitutively activate the JAK2-STAT5 signalling pathway. These results are in agreement with a previous report that describes R683G as an activating mutation.[42]

To further explore the functional consequences of the two activating *JAK2* mutations, we assess whether these mutations are able to drive the proliferation of BaF3-EpoR cytokine-dependent cell line. To this end, we transduced BaF3-EpoR cells and selected for stable expression of wild-type or mutant-*JAK2*. As expected,[37,39,41] the R683G mutation can drive itself the proliferation of BaF3-EpoR cells, conferring cytokine independence as assessed by cytokine withdrawal assays for at least four days in cytokine-starving conditions. Proliferation of BaF3-EpoR cells in the absence of exogenous cytokines was not observed in the case of H574R or the wild-type JAK2 protein, but the number of viable cells was significantly higher in BaF3-EpoR cells expressing the H574R mutant with regard to cells expressing the wild-type gene, even after 4 days in the absence of cytokine (Figures 2b and c).

In cytokine-starving conditions, only R683G-JAK2 remains phosphorylated and is able to induce STAT5 phosphorylation (Figure 2d). This capacity can be sustained for at least 4 days (Supplementary Figure S2). Furthermore, H574R and R683G showed higher levels of JAK2 phosphorylation than wild-type JAK2 in response to Epo stimulation after starving conditions (Figure 2d).

JAK2 activation might be enhanced by hypermethylation of the negative regulator SOCS3

Because epigenetic inactivation of three negative regulators of JAK2 (*SHP1*, *SOCS1* and *SOCS3*) have been reported in many tumors,[17,36,41,43–47] we determined the levels of CpG methylation at the promoter regions of these genes in the three T-LBL samples exhibiting missense mutations and the TEL-JAK2 translocation (Figure 3).

Methylation-specific PCR showed that there was no significant aberrant hypermethylation in the CpG islands at the promoter region of *SHP1* and *SOCS1* genes in any T-LBL sample. However, we found aberrant hypermethylation of *SOCS3* promoter in the three samples (Figure 3b). Furthermore, bisulfite genomic sequencing of 10 clones per sample confirmed significant hypermethylation of *SOCS3* with methylation densities ranging from 45.2 to 94.8% (Figure 3c; Supplementary Table S3).

To assess whether *SOCS3*, *SOCS1* and *SHP-1* genes were methylated in the T-LBL cases with intact JAK2 gene, we have also evaluated gene methylation of these genes in the remaining T-LBL samples. Methylation-specific PCR analysis revealed that (i) *SOCS1* is not hypermethylated in any T-LBL sample; (ii) SHP1 is hypermethylated in only two lymphomas exhibiting intact *JAK2* sequences (685 and 038); and (iii) aberrant hypermethylation of *SOCS3* is a primary event in T-LBL development. Using a cutoff of 20% methylation density, 7/10 lymphomas also exhibit significant hypermethylation of *SOCS3* promoter. These results indicate that aberrant hypermethylation of *SOCS3* is not dependent on the mutational status of *JAK2* (Supplementary Figure S3).

To further validate whether promoter DNA hypermethylation is involved in deregulating the expression of *SOCS3*, Jurkat cells (which exhibit a high methylation density of about 93.7%) were treated with 5-aza-2′-deoxycytidine. Significant demethylation of *SOCS3* promoter was observed after treatment with 5-aza-2′-deoxycytidine, which brings about *SOCS3* upregulation at the mRNA level. Treatments with trichostatin A, a histone deacetylase inhibitor, produced only modest but significant increases. However, the combination of both drugs seemed to be the most successful treatment (Figure 4). These results were confirmed using the Molt-4 cell line (Supplementary Figure S4). So, we propose that the aberrant promoter hypermethylation of *SOCS3*

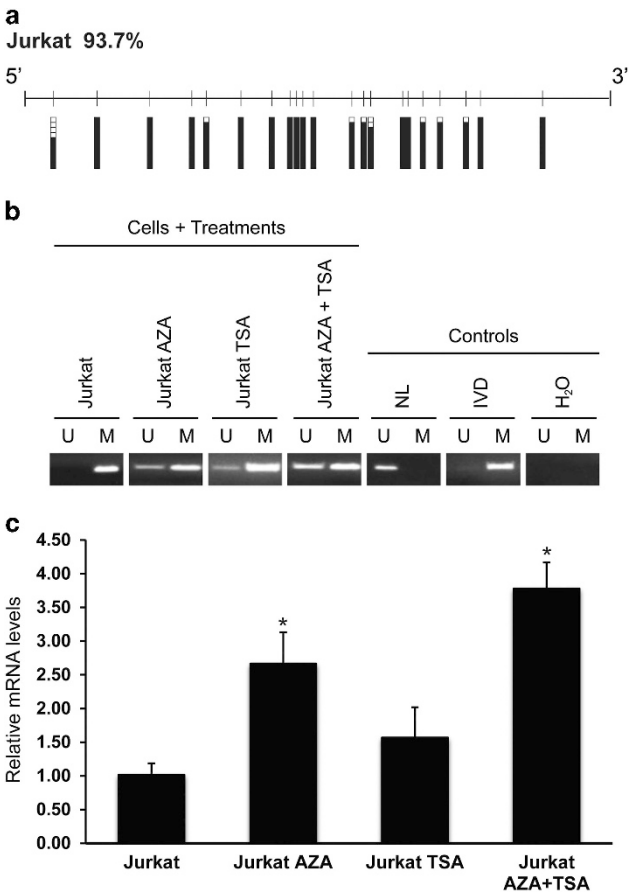


Figure 4. Methylation analysis of *SOCS3* in Jurkat cells. (**a**) Methylation profile of the CpG island. The presence of methylated black squares) or unmethylated (white squares) CpG sites is indicated in 10 sequenced clones. Methylation density is indicated as the percentage of methylated CpG sites. (**b**) Methylation-specific PCR of untreated cells and cells treated with 5-aza-2´-deoxycitidine (AZA) and trichostatin A (TSA). M, methylated; U, unmethylated; NL, Normal lymphocytes; IVD, *in vitro* methylated DNA. (**c**) Levels of mRNA expression of *SOCS3* in treated and non-treated cells. Quantitative real-time RT-PCR analysis of *SOCS3* transcripts showed significant differences between treated and non-treated cells ($^*P < 0.05$).

we have detected in T-LBL samples might be contributing to a partial inactivation of this gene and, consequently, to enhance JAK2 activation in these primary lymphomas.

Next, we evaluated whether there exists a good match between SOCS3 protein expression and methylation density at the promoter region of this gene in primary T-LBLs. Remarkably, some tumors exhibiting high levels of hypermethylation (238 and 734) also showed a very significant reduction of SOCS3 protein. However, other mechanisms than promoter hypermethylation have to be considered in other cases (Supplementary Figure S5).

As previous reports had demonstrated high pSOCS3 levels in specific mutant JAK2-bearing samples from myeloproliferative neoplasms and in PCM1-JAK2-positive cells,[32,48] we have also studied the levels of endogenous SOCS3 tyrosine phosphorylation induced by wild-type and two activating mutant JAK2 variants (H574R and R683G). These analyses were performed in two different experiments using BaF3-EpoR and γ2A cells according to the protocols described by Elliot *et al.*[32] However, we found no significant differences in the levels of phosphorylated SOCS3 that can be attributed to these JAK2 mutations (see Supplementary Figure S6)

All missense mutations induced a significant increase of LMO2 expression

In addition to activating the canonical cytokine-signaling pathway, a recent study demonstrated a non-canonical function for JAK2 activation as epigenetic regulator.[24] In this article, the authors demonstrated that a significant proportion of JAK2 is present within the nuclei of hematopoietic cells, which is able to increase the expression of LIM domain Only 2 *(LMO2)* gene. Because *LMO2* is a key oncogene operating in a fraction of T-cell lymphoblastic leukemia/lymphomas,[25–28] we investigated the possibility of a mechanistic relationship between *JAK2* mutations and the expression of *LMO2* in primary T-LBLs. Interestingly, the three T-LBL samples harboring missense mutations or the TEL-JAK2 translocation (460, 734 and 829) exhibited significant increases in *JAK2* and *LMO2* expression.

To experimentally evaluate the relationship between *JAK2* mutations and *LMO2* expression, we analyzed (by qRT-PCR) the levels of *LMO2* in γ2A cells expressing with wild-type or mutant *JAK2* variants. Notably, all missense mutations were capable of inducing significant increases in the expression of *LMO2* when compared with the wild type. As there are no apparent differences in JAK2 protein expression between wild-type and mutant JAK2 variants, this factor should not disturb the interpretation of the data and their statistical significance (Figure 5b). Confocal immunofluorescence in γ2A cells expressing wild-type or mutant *JAK2* variants revealed a significant increase in the number of cells exhibiting nuclear expression of mutant JAK2 proteins (Figures 6a and b). This suggests that increased *LMO2* expression may be consistent with a greater presence of JAK2-mutant protein into the nucleus. Notwithstanding, the type of mutation is another factor to take into account, because no perfect matching can be reached when the percentage of γ2A cells expressing nuclear JAK2 and the levels of *LMO2* expression are compared (Figures 5b and 6b).

Immunohistochemistry analyses were performed with fresh frozen tissue sections of two primary T-LBLs, one harboring wild-type JAK2 (238) and the other bearing activating JAK2 mutations (460). Remarkably, the presence of JAK2 into the nucleus was more pronounced in lymphoma 460 (8.9% of cells) than in lymphoma 238 (3.1%) (Figure 7).

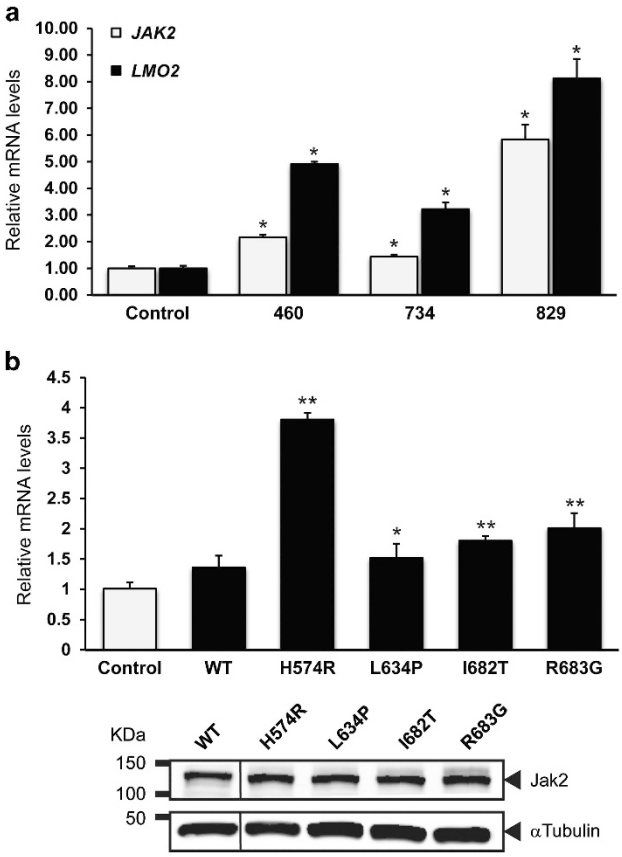


Figure 5. qRT-PCR analysis of *JAK2* and *LMO2* expression. (**a**) The mean mRNA values of *JAK2* and *LMO2* in T-LBLs harboring JAK2 genetic alterations were normalized to those of a pool of fetal thymuses. Data represent three independent replicates. Error bars represent the s.d.; $*P < 0.01$. A representative western blot of JAK2 can be seen under the bar chart. (**b**) The mean mRNA values of *LMO2* in γ2A cells transiently transfected with mutant JAK2 variants were normalized to that of wild-type JAK2-expressing cells. Data represent three independent replicates. Error bars represent the s.d.; $*P < 0.05$ and $**P < 0.01$.

DISCUSSION

At present, more than 30 mutations in the JH2 domain of *JAK2* gene have been shown to be related with hematological diseases,[49] but the involvement of JAK2 in T-LBL is reduced to a rare rearrangement resulting in the constitutively active TEL-JAK2 fusion oncoprotein.[12] In the present article, we identified a TEL-JAK2 translocation in one adult patient exhibiting an immature T-LBL and validate the presence of four missense *JAK2* sequence variants in the transcriptome of T-LBLs.

As the TEL-JAK2 fusion we found in patient 829 results from the direct fusion of the Kinase (JH1) domain of *JAK2* and the 5´ region of the *TEL* gene (which contains an oligomerization domain), this rearrangement should induce constitutive activation of the JAK-STAT signaling pathway. In support of this notion, we found high levels of phospho-STAT5 in this tumor (Figure 1). Noteworthy, similar translocations reported in other tumors give rise to the expression of an oncogenic protein that leads to constitutive activation of the JAK2-STAT signaling pathway[50] and is able to induce cytokine-independent growth in the murine hematopoietic cell line BaF3-EpoR.[12] Furthermore, bone marrow transplantation in mice using hematopoietic cells exogenously overexpressing TEL-JAK2 results in mixed myeloproliferation and T-cell lymphoproliferation.[51] Interestingly, the TEL-JAK2 fusion-bearing patient 829 suffers a ProT-lymphoblastic lymphoma that exhibits a mixture of T-cell lymphoid and myeloid markers (CD3+, CD1a+, MPO+; Supplementary Table S1).

In agreement with previous reports on other hematological malignancies, *JAK2* mutations found here accumulated in the

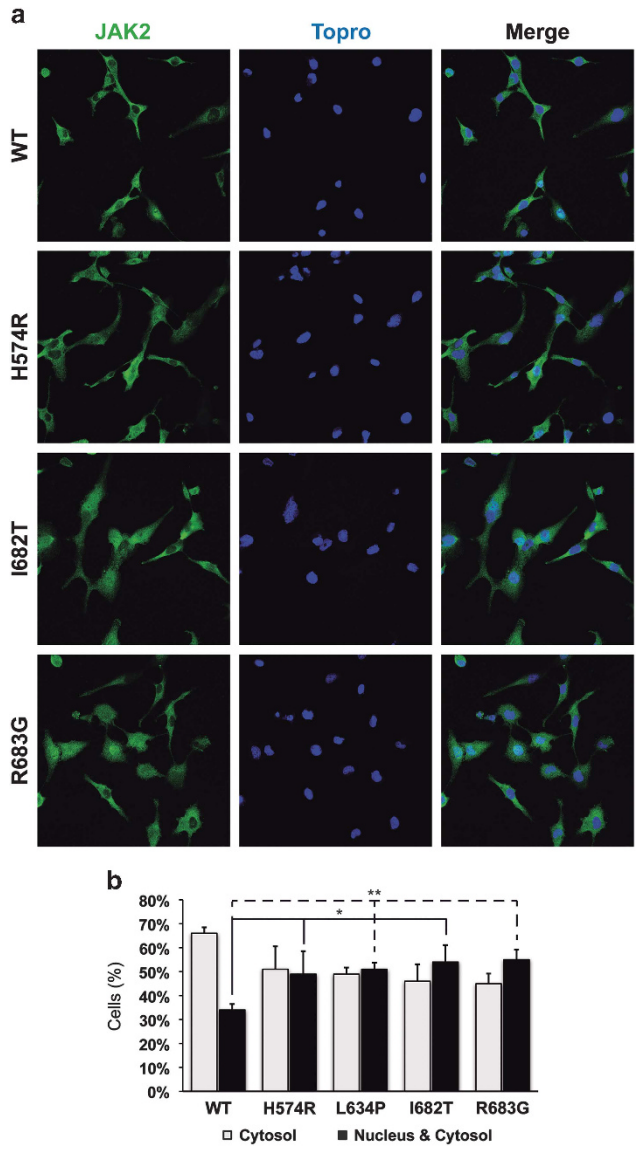


Figure 6. Subcellular localization of JAK2 in γ2A cells transiently expressing wild-type or mutant JAK2 variants. (**a**) Confocal immunofluorescence microscopy images identify JAK2 (green) in γ2A cells expressing wild-type JAK2 or H574R, I682T and R683G JAK2 mutants, as representative. Nuclear staining was determined by Topro (blue). (**b**) Histogram representing the quantification of γ2A cells positive for JAK2 cytosolic localization and positive for JAK2 nuclear and cytosolic localization, in percentage. γ2A cells transiently expressing wild-type and every mutant JAK2 variant have been analyzed; note that all mutants (H574R, L634P, I682T and R683G) were significantly more localized in the nucleus than the wild-type JAK2. Error bars represent the s.d.; $*P < 0.05$ and $**P < 0.01$.

pseudokinase domain (JH2), which functionally prevents the activation of the kinase domain (JH1).[52] Two of the four missense mutations had not been previously described for *JAK2*. Activating R683G mutation has been previously described in B-cell acute lymphoblastic lymphoma,[37,39,41] thus serving as a point of reference in our functional analyses. Notably, we have not identified activating mutations at exon 14, where a gain-of-function substitution (V617F) that is able to abrogate JH2 activity in myeloproliferative neoplasms[52,53] has been described in more than 90% of patients with polycythemia vera.[14–16] On the other hand, we have identified novel sequence variants at exon 13 and exon 15.

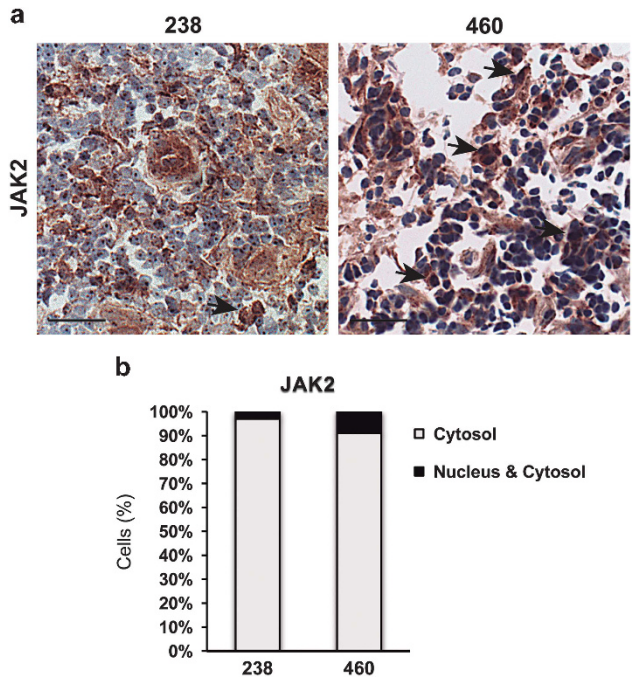


Figure 7. JAK2 nuclear localization in primary T-LBL samples. (**a**) Sample 238 with intact JAK2 (left) and sample 460 harboring several JAK2 mutant variants (right). Arrows indicate cells with nuclear expression of JAK2. Magnification bar, 25 μm. (**b**) Histograms showing the percentage of cells with cytosolic and nuclear localization of JAK2.

It should be noted, however, that all *JAK2* mutations found are accumulated in two pediatric patients (460 and 734) and that both lymphomas exhibited three or more different variants with similar pathogenic properties, suggesting sub-clonal heterogeneity (Figure 1 and Supplementary Figure S1). Therefore, one can speculate that an important fraction of lymphoma cells on each mutated T-LBLs might be addicted to the activation of JAK2 pathway but not to any particular mutation. In addition, the fact that the most common mutations (R683G and H574R) appeared in a limited number of JAK2 transcripts, could explain that previous reports have identified mutations in *JAK1* and *JAK3* but not in *JAK2*.

In the case of lymphoma 460, R683G-JAK2 mutant is able to activate the JAK2-STAT pathway in a constitutive manner, to confer BaF3-EpoR cell line cytokine-independency and to induce a significant increase of *LMO2* expression in transfected γ2A cells. The other *JAK2*-mutation (I682T) is able to increase the expression of *LMO2* exclusively. In the case of lymphoma 734, mutant H574R is able to activate the JAK2-STAT5 pathway, to drive the proliferation of the cytokine-dependent cell line BaF3-EpoR and to induce significant increase in *LMO2* expression. Thus, we could hypothesize that different combinations of *JAK2* mutations are acting in concert to stimulate T-cell lymphoma development by two different mechanisms: the activation of the canonical cytokine-signaling pathway and the activation of *LMO2*, a key oncogene in hematological malignancies. Taken together, these results indicate that inhibition of JAK2 signaling using JAK pan-inhibitors is a rational target for therapeutic intervention of T-LBLs exhibiting activated JAK2.

On the other hand, the inhibition of *SOCS3* seems to facilitate tumor formation and growth in multiple human cancers.[35,54,55] Deletion of the *SOCS3* gene has been reported in liver parenchymal cells that promote hepatitis-induced carcinogenesis;[23] also, aberrant hypermethylation of CpG islands in *SOCS3* associated with reduced expression has been observed

in various human cancers including several lymphoid malignancies.[56] Interestingly, we found aberrant hypermethylation at the promoter region of *SOCS3* in the two T-LBL samples exhibiting *JAK2* mutations and in the sample with the TEL-JAK2 fusion protein. We therefore support that epigenetic inactivation of *SOCS3* might be a modulator event that should be considered in T-LBLs. Interestingly, the epigenetic profile of primary lymphomas is similar to that exhibited by Jurkat cells (Figure 4). Because treatment of Jurkat cells with 5-aza-2′-deoxycytidine (5′-aza, decitabine) and histone deacetylase inhibitors significantly increase the expression of *SOCS3*, our data support that T-LBL patients with hypermethylated *SOCS3* might benefit from additional treatments with DNA methylation inhibitors, in combination with histone deacetylase inhibitors. Furthermore, because pharmacologic agents that block SOCS3 expression and function may sensitize glioblastom multiform cells to ionized radiation,[57] the use of these drugs might ultimately improve the effectiveness of radiotherapy in treating T-LBLs exhibiting activation of the JAK-STAT signaling pathway.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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